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# The Inhibitory Control of Prothoracic Gland Activity by the Neurosecretory Neurones in a Moth, *Mamestra brassicae*

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**ABSTRACT**—The change in both the ecdysteroid titers in the haemolymph and the amount of ecdysteroids released from the prothoracic gland (PTG) *in vitro* during larval development were determined in *Mamestra brassicae*. These showed a clear inverse relationship to the efferent electrical activity in the PTG nerves, even in the detailed changes in each day during development. An exception was the final day of the last instar. This result suggests that, as well as the activating effect of prothoracicotropic hormone (PTTH), the PTG innervating neurosecretory neurones are possibly involved in the delicate inhibitory adjustment of the amount of ecdysteroids in the haemolymph. Here, the simultaneous determinations of the amount of ecdysteroids released from PTG and of the electrical activity of nerves by using the isolated preparation *in vitro*, provides direct evidence of the inhibitory effect of PTG innervating nerve on the PTG.

## INTRODUCTION

Various types of PTG innervating neurones were described in the previous paper [1]; both histological and electrophysiological studies showed that they are neurosecretory. The electrical activities of one of PTG innervating nerves shows the remarkable changes during the course of development, its firing frequency varying inversely with the ecdysteroid titers in the haemolymph as examined by Agui and Hiruma [2]. These results and those from surgical experiments [3] have led to the suggestion that the function of this PTG innervating nerve is to inhibit the PTG.

To analyse accurately this inverse relationship between the electrical activity of the PTG innervating nerve and ecdysteroid titer in the haemolymph, it is important to reexamine the haemolymph ecdysteroid titers by using the same animals from our strain and reared under the same condition as was used for the measurement of the electrical activity. Also, it is better to use isolated preparations for determination of both the released ecdysteroids from PTG and the electrical activity of PTG innervating nerves. Therefore, the time change of the ability to synthesize ecdysteroid in the isolated PTG *in vitro* was determined. In this paper, the simultaneous determinations of the amount of released ecdysteroid from PTG and of the electrical activity of the PTG innervating neurones were made to obtain direct evidence for the nervous inhibition of the PTG activity.

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## MATERIALS AND METHODS

Larvae of *Mamestra brassicae* were reared on artificial diet [4] at 23°±1°C under a long day photoperiod (16L, 8D). The time course of development of the last instar larva was the same as described in the previous paper [1]. The first day after ecdysis was designated day 0. Female larvae were used throughout this experiment.

Ecdysteroid titers in the haemolymph were determined by radioimmunoassay (RIA). Haemolymph collection was made four times in each day of larval development at 0:00, 6:00, 12:00 and 18:00, the middle of dark period being taken arbitrarily as 24:00 (0:00). Ten $\mu$l haemolymph was taken from each larvae, by making a small cut

on a proleg, and introduced into 400 μl methanol. After mixing vigorously, this sample was centrifuged at 3000 g for 20 min, methanol in the supernatant was evaporated at 40°C, and the sample was stored frozen at −20°C. Before starting the RIA procedures, the dried sample was dissolved in 100 μl borate buffer (pH 8.4). If necessary this solution was diluted to one tenth. Forty μl of this solution were used as the biological sample in RIA.

The amount of ecdysteroids released from PTG *in vitro* was also determined by RIA. PTGs were extirpated from the larvae of various stages of last instar. The isolated PTGs were kept immersed in 100 μl Grace's medium for about 1 hr. The necessity of the immersion of PTG in the culture medium will be described later. The PTGs were then transferred and incubated usually for 2 hr in 100 μl new Grace's medium dropped on each compartment of an acrylic culture well (Falcon 3047, Becton Dickson & Co.). The culture wells were placed in a container which was filled with a gas mixture of $O_2$ 95% and $CO_2$ 5%, saturated with water vapor and kept at 23° ± 1°C. After the incubation, 40 μl of the culture medium was collected and used as the biological sample in RIA. The culture method for the isolate ganglion-PTG preparation used for studying the ecdysteroid synthesis under the influence of PTG innervating nerves will be described later in the corresponding part of results.

RIA for ecdysteroids was performed according to Borst and O'Conner [5] using [23, 24-$^3$H] ecdysone (ca. 70 Ci/mmol, New England Nuclear). The antiserum of ecdysterone (20-hydroxyecdysone, Rhoto Pharmaceutical, Osaka) was prepared in collaboration with Prof. E. Ohnishi, Nagoya University, Prof. N. Ikekawa, Tokyo Institute of Technology and Dr. K. Ozawa, Megro Institute (Osaka). Practical steps of RIA procedures were based on and modified from Bollenbacher *et al.* [6] which presented the systematic descriptions for the study of the synthetic ability of ecdysteroids by PTG *in vitro*. Antiserum was diluted to 1/3200 with 10% rabbit serum dissolved in the borate buffer so that about 50% of the radioactivity of labelled ecdysone was bound in the absence of unlabelled ecdysone. The labelled ecdysone (ca. 8 nCi) in 80 μl buffer and 50 μl antiserum solution was added to each of a pair of 40 biological samples described above. Duplicated assays were performed for each sample. The mixture was stored at 4°C for 12–16 hr. After precipitation using ammonium sulfate, the precipitate was dissolved in 50 μl distilled water and 400 μl scintilation solution (ACS II, Amersham) was added. Radioactivity was determined using Packard scintilation counter (model 240 CL/CLD).

The standard curve for ecdysone (Sigma) was log-linear between 10 and 200 pg and that for 20-hydroxyecdysone (Rhoto Pharmaceutical) between 30 and 1000 pg. Therefore, in this experiment, the binding curve for ecdysone was exclusively used for both determinations of the haemolymph ecdysteroid and the secreted ecdysteroid from the PTG *in vitro*. All data were expressed as ecdysone equivalent, because the determinations in this experiment by RIA were based on the cross reactivity of the prepared antiserum with ecdysone, 20-hydroxy-ecdysone and other ecdysteroids.

## RESULTS

### *Haemolymph ecdysteroid titers*

Figure 1 shows the change in haemolymph ecdysteroid titer during development of the last instar larva. To show clearly the small change in ecdysteroid titer, the values from day 1 to day 7 were plotted separately with five fold magnification in the vertical scale. There are small peaks at each light period of day 3-6 which show gradual increase in magnitude with the progress of the development. It is difficult to assess directly which peak of haemolymph ecdysteroids is the stimulant for the appearance of transparent epidermis or for wondering behaviour to the ground. The highest peak prior to the pupal ecdysis was observable at 18:00 of day 8. Quantitative comparison between ecdysteroid titers in the haemolymph and the electrical activities of PTG innervating nerves during development will be given at the later section of results.

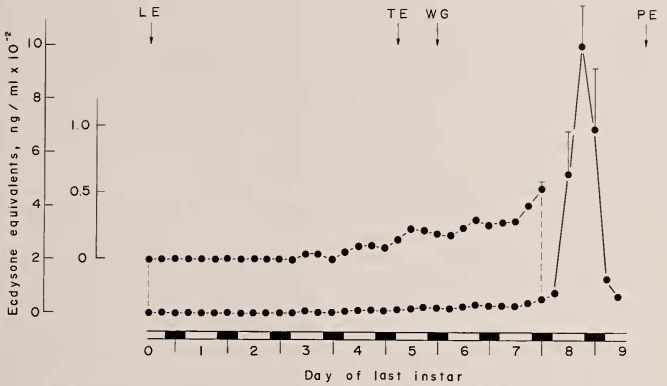


FIG. 1. Ecdysteroid titers in the haemolymph of *Mamestra brassicae* during the development of last instar larva. Each point is the mean (±SEM) of ten measurements. The following developmental stages are indicated by arrows: LE, larval ecdysis; TE, appearance of transparent ventral epidermis; WG, wandering to the ground; PE, pupal ecdysis. Light and dark periods in each day are shown at the lower part of the figure. See text for details of the representations of ecdysteroid titer from day 1 to day 7.

*Released ecdysteroids from PTG in vitro*

Time course of ecdysteroid synthesis by PTG *in vitro* was determined to find a convenient incubation condition for the study of the change in ability of the ecdysteroid synthesis during development. PTGs were extirpated at 9:00–11:00 from day 8 last instar larva and then incubated in Grace's medium. The ecdysteroid titer determinations in Figure 1 suggest that the isolated PTG from the larvae of this stage would be sufficiently potent to produce the ecdysteroids under the influence of

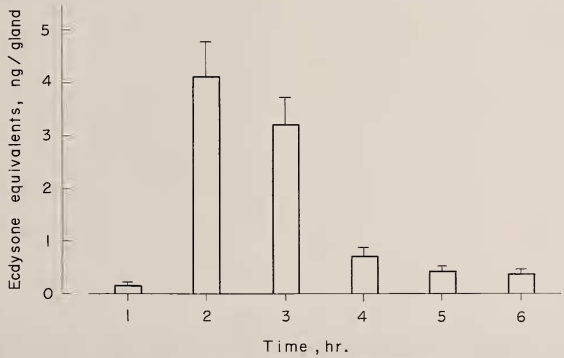


FIG. 2. Time course of the ability to synthesize ecdysteroid after incubation of PTG in the Grace's medium. Each point is the mean (±SEM) of ten measurements.

the prothoracicotropic hormone (PTTH) in the haemolymph.

The amount of ecdysteroid released from PTG into the external medium every hour after the start of incubation was determined by RIA as shown in Figure 2. The ecdysteroid synthesis shown in this figure is limited to low values in the first 1 hr and elevated significantly in the succeeding 2 hr. The rate of ecdysteroid synthesis shows the marked decrease at 4 to 6 hr after the incubation, probably due to washing out the PTTH by the repeated changes of the external medium. The exact reasons for the limited synthesis in the first hour after incubation were difficult to find here physiologically.

In consideration of the time course of ecdysteroid synthesis shown in Figure 2, the following procedure was chosen for the determination of the change in ability of ecdysteroid synthesis during development. After incubation in the culture medium for about 1 hr, the PTGs were transferred and incubated in new Grace's medium for 2 hr, the external medium being used as a samples for RIA determination. In this series, PTG isolations were made at 9:00–11:00 from the larva of each development stage, and consequently incubated for 2 hr from 10:00–12:00 to 12:00–14:00.

The results in Figure 3 shows that a measurable amount of ecdysteroid synthesis is observable at day 3 and the values are increased by day 5 where the transparent epidermis was visible. The amounts of ecdysteroid synthesis remained fairly stable for 3 days from day 5 to day 7 increased significantly at day 8.

*Comparison of ecdysteroid synthesis and electrical activity of nerves to PTG*

It is important to compare three results; the ecdysteroid titers in the haemolymph (Fig. 1); ecdysteroid synthesis by PTG *in vitro* (Fig. 3); and electrical activities of the PTG innervating nerve (Fig. 8 in the previous paper [1]) during the larval development. These results are superimposed in Figure 4, where only data in Figure 1 obtained at 12:00 are plotted for better comparison.

There are clear parallels between the haemolymph ecdysteroids and the ecdysteroids synthesized *in vitro,* except that the rate of increase of the haemolymph ecdysteroids from day 7 to day 8 is about thirty times but only about two times in the latter case. This will be discussed below. The critical comparison of the absolute

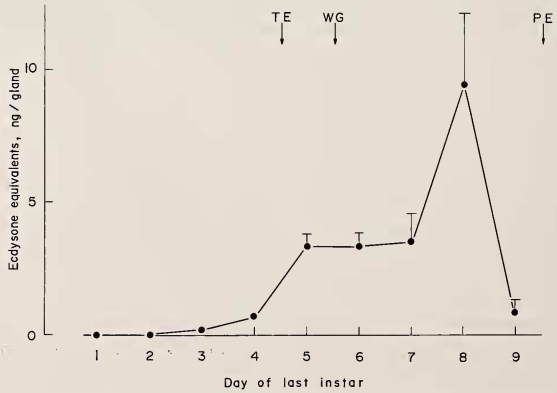


Fig. 3. Ecdysteroid synthesis by PTG *in vitro* during the development of last instar larva. Each point is the mean (± SEM) of ten measurements. Refer to Fig. 1 for explanation of the developmental stages shown at the upper part of the figure.

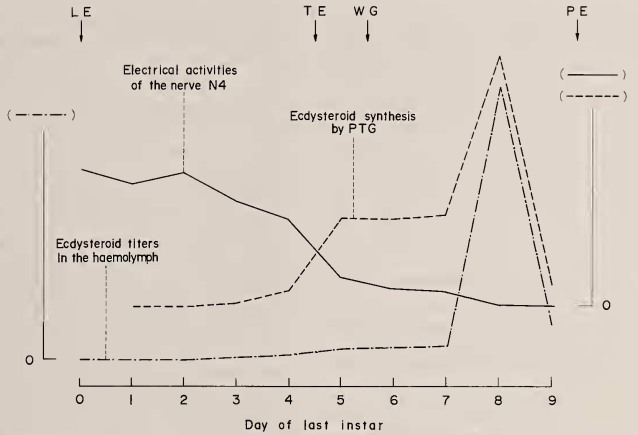


Fig. 4. Superimposed representation of ecdysteroid titers in the baemolymph (Fig. 1), ecdysteroid released from PTG *in vitro* (Fig. 3) and electrical activities of PTG innervating nerve N4 [1]. Refer to Fig. 1 for explanation of the developmental stages shown at the upper part of the figure.

values determined in both cases is difficult because the amount of haemolymph ecdysteroids cannot be compared directly with the values obtained using PTG preparation *in vitro*. In this connection, Warren *et al.* [7] published critical studies on the PTG activity of *Manduca sexta* with an important discussion of the results obtained from the RIA of released ecdysteroids in the culture medium from the isolated PTG.

Careful comparison in Figure 4 of the ability to synthesize ecdysteroid *in vitro* and the electrical activities of PTG innervating nerves in the isolated preparation indicate a clear inverse relationship even in the precise changes in each day during the development, day 9 being an exception. Detailed descriptions of the change of electrical activities each day have been given in the previous paper [1].

*Inhibitory action of nerves to PTG on the ecdysteroid synthesis by PTG*

The inverse relationship between electrical activity in nerve N4 to PTG and ecdysteroid synthesis by the PTG, as was demonstrated in the previous section of this paper as well as in the previous paper [1], suggest that the activity of the nerve N4 is inhibitory to the ecdysteroid synthesis or release at PTG. This suggestion can be tested directly by simultaneous determination of released ecdysteroid from PTG and of electrical activities of nerves in the same preparation *in vitro*.

The preparations were isolated as the ganglionic chain from brain to mesothoracic ganglion connected with PTG by the nerve N4. In the first, the nerve N4 in the isolated preparation was placed on the oil gap as shown in Figure 1A of the previous paper [1] and the ganglion and the PTG were immersed separately in the two Grace's medium pools of the gap chamber. These oil-gap chambers were placed in a gas mixture as described above.

However, unexpected troubles occurred for two reasons. At first, after several min immersion of PTG in Grace's medium, there was enhanced afferent activity in the nerve N4 followed by the disappearance of the efferent activity generated at the prothoracic ganglion and conducted to PTG. Analysis of this phenomena will be made in the following paper. This problem was solved by replacing the physiological saline described in the

previous paper [1] with a K-rich saline with 70 mM KCl. With this medium, the efferent activity continued fairly well during at least half the period of PTG incubation in the Grace's medium.

Secondly, the rate of ecdysteroid synthesis by PTG in the oil-gap chamber was drastically reduced. This was due to chemical and/or physical disturbance by the paraffin oil on the synthetic activity of PTG and also to the difficulty of supplying oxygen to PTG in the incubation pool. This problem was reduced by using white vaseline instead of paraffin oil. Although the rate of ecdysteroid synthesis was reduced to about 1/5 in comparison with the case of the usual organ culture described above, the values obtained in the same experimental condition were fairly uniform.

The preparations were obtained from day 5 last instar larva. After the isolated preparations were arranged on the gap chamber in the improved manner and kept for 1 hr, the external medium was replaced by the new Grace's medium and the samples for RIA were collected after 2 hr incubation. Nervous activity was monitored during incubation period. For the control determinations, contralateral PTGs with the connection of the nerve N4, severed just in front of prothoracic ganglion, were isolated from the same specimen used for the test above, and placed in the similar manner on the individual gap chambers. The efferent electrical activity was not routinely recorded from the nerve of this preparation.

The results are shown in Figure 5 where the rate of inhibition is expressed as a ratio divided by the value obtained from the control experiment. For comparison, ratios between right and left of a single pair of PTGs isolated from the connection with ganglia are also plotted. Thirty animals were used for each comparison; to help the immediate comparison in the figure the degree of inhibition was expressed by the number of animals showing the values which would fit the corresponding ranges of inhibition ratio. This figure shows that the action of the nerve N4 is inhibitory to the ecdysteroid synthesis at PTG. Although the small number of the preparations showed no inhibitory effect, this probably occurred by the injurious

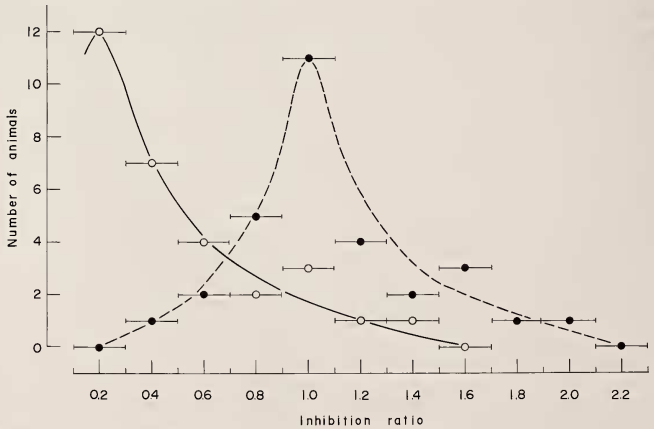


Fig. 5. Inhibitory effects of PTG innervating nerves on the rate of ecdysteroid synthesis by PTH. The rate of inhibition obtained by comparing the abilities of ecdysteroid synthesis in preparations both with and without PTH innervating nerves. The results obtained by the above determinations are plotted as white circles and those obtained by comparing the synthetic activities in both right and left PTGs of a single specimen as black circles. See the text for the exlanation of the inhibition ration.

effects on nerves or nerve junctions with PTG in the case of setting the isolated preparation on the gap chamber.

## DISCUSSION

Based on the results suggested from the electrophysiological determinations in the first paper [1] of this series, the present work using RIA techniques was intended to establish the real function of the nerves to PTG with respect to the PTG activity. Direct determinations of the amounts of synthesized ecdysteroid under the influence of nerve N4 to the PTG clearly showed the inhibitory function of the PTG nerves on the PTG. This activity is assumed to be neurosecretory as described in the previous paper [1]. However, it is not yet clear whether the neurosecretory substance released into the PTG inhibits the synthetic or releasing process at PTG.

The high afferent electrical activity observed in nerve N4 shortly after the immersion of the isolated nervous system in Grace's medium and the following reduction in efferent activity is similar to changes already noticed during measurements of efferent activity during development. Here it was found that the firing frequency was greatly influenced by the chemical composition of the external medium [1]. Although the details of these phenomena will be described in the following paper, the results described above indicate that the main feedback machinery for monitoring the chemical condition of haemolymph must be contained in the ordinary isolated preparation of the nervous system.

The electrical activity of nerve N4 was found to show the remarkable changes with the progress of the larval development exactly related to the change in ecdysteroid synthetic or releasing activity at PTG. This result suggests that, as well as the activation effect of PTTH, the neurosecretory neurones, which directly innervate PTG, are involved in the precise adjustment of the amount of haemolymph ecdysteroids. These may regulate the appearance of the various important events of the development, like the wandering behaviour as has been analyzed in the lepidopterous larvae by Gilbert *et al.* [8], Dominick and Truman [9], Fujishita *et al.* [10]. The exception observed at the final day of larval development to pupa probably means that the drastic decrement of the haemolymph ecdysteroids is solely governed by the chemical inactivation of ecdysteroids in the haemolymph. Similarly, the elevation to the extremely high level of haemolymph ecdysteroid titers just before pupal ecdysis (Fig. 1) may not be the consequence of regulation by the PTG innervating nerves.

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